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Does the 48-hour BH4 loading test miss responsive PKU patients?

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ABSTRACT

Background: Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism. Besides dietary treatment, some patients are responsive to and treated with tetrahydrobiopterin (BH4). Our primary objective was to examine whether the 48-hour BH4 loading test misses BH4-responsive PKU patients. Secondary, we assessed if it would be beneficial to 1) use a cut-off value of 20% Phe reduction instead of commonly used 30%, and 2) extend the loading test to 7 days.

Methods: 24 patients with a 20–30% decrease of blood Phe levels during their initial 48-hour BH4 loading test or at least one mutation associated with long-term BH4 responsiveness, were invited to participate. 22 of them underwent the 7-day BH4 loading test. During the BH4 loading test, BH4 was administered orally once daily for 7 days (20 mg/kg/day). Blood samples on filter paper were collected at 13 time points. Potential BH4 responders (≥20% decrease in blood Phe concentrations at ≥1 moment within the first 48 h or ≥30% at ≥1 moment during the entire test) underwent a treatment trial to assess true long-term responsiveness (≥30% decrease of Phe levels compared to baseline and/or ≥50% increase in natural protein tolerance in accordance with the Dutch guidelines before 2017). The duration of the treatment trial varied from 2 to 18 months.

Results: Of the 22 patients who completed the 7-day BH4 loading test, 2 were excluded, 8 had negative tests and 12 were considered to be potential BH4 responders. Of these 12 potential BH4-responsive PKU patients, 5 turned out to be false positive, 6 true-responder and 1 was withdrawn.

Conclusion: Even though the 48-hour BH4 loading test has proven its efficacy in the past, a full week may be necessary to detect all responders. So, if blood Phe concentrations during the 48-hour BH4 test shows a clear tendency, but not sufficient decrease, a full week (with only measurements each 24 h) could be offered. A threshold of ≥20% decrease within 48 h is not useful for predicting true BH4 responsiveness.

1. Introduction

Phenylketonuria (PKU; McKusick #261600) is an autosomal recessive inborn error of phenylalanine (Phe) metabolism, caused by a deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH). PAH converts Phe into tyrosine (Tyr) requiring the co-substrate tetrahydrobiopterin (BH4). Left untreated, PKU results in high blood Phe levels, causing intellectual disability, seizures, and behavioural disturbances [1].

The main treatment is a diet low in natural protein in combination with Phe-free L-amino acid supplements (AAS). Some patients are responsive to and treated with BH4, prescribed as sapropterin dihydrochloride. In these patients, BH4 acts as a pharmaceutical chaperone that increases residual PAH activity. Patients who are responsive are able to increase their natural protein intake and/or to reduce their blood Phe levels [2,3].

The gold standard for determining whether patients are responsive to BH4 is to treat all patients with BH4 for months and evaluate if blood Phe levels decrease and/or patients' natural protein tolerance increases. However, this is time-and cost-consuming. Therefore, determination of potential BH4 responsiveness is usually done by genotype and/or BH4 loading tests [4]. The duration of short-term loading tests varies from 48 h in Europe [5] to 28 days in the USA [6]. In the Netherlands, BH4 has been available since 2009. Since then most PKU patients underwent

Abbreviations: AAS, Phe-free L-amino acid supplements; BH4, tetrahydrobiopterin; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, Phenylketonuria; Tyr, tyrosine

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a 48-hour BH4 loading test. During this test, blood Phe was analysed for 48 h (T = 0, 8, 16, 24, 48 h) after the administration of a single daily dose of 20 mg/kg BH4 with a second dose after 24 h. A patient was considered to be a potential BH4 responder when a reduction in blood Phe of ≥30% compared to baseline was seen at ≥1 of the time points. Potential BH4 responders then underwent a treatment trial of several weeks or months where natural protein intake, BH4 dosing and AAS were adjusted. Until the publication of the first European PKU guidelines [4,5], patients were considered to be true-responders when they were able to decrease their Phe levels with ≥30% compared to baseline and/or increase their natural protein tolerance with ≥50% or 4 g during this treatment trial.

Data of a Dutch cohort show that the positive predictive value (PPV) of the 48-hour BH4 loading test is 87% [7]. Unfortunately, these data do not allow us to analyse the negative predictive value and thus whether there are any false-negative tests. There is evidence that some patients take longer than 48 h to respond to BH4 [8–10]. Also, the 30% blood Phe decrease threshold is arbitrary [11] and possibly a 20% reduction is enough to predict a clinically relevant response in some cases [12]. A recent survey showed that the 20% cut-off is even used as the cut-off for true-responsiveness in 17 countries [13].

Therefore, the primary objective of this study was to assess whether the 48-hour BH4 loading test misses BH4-responsive patients. Secondary to this, we examined 1) if the threshold of 30% in 48-hours test should be lowered to 20%, and 2) if the current 48-hour test should be extended to 7 days to detect more BH4-responsive patients.

2. Material and methods

This study was conducted between September 2016 to August 2019 in two centres: UMC Groningen and Radboudumc Nijmegen. The medical ethical committee of UMC Groningen ascertained the protocol as routine care for PKU patients treated in the Netherlands. The design of the study was a prospective cohort study consisting of two parts: the 7-day BH4 loading test and the BH4 treatment trial.

2.1. Inclusion criteria

Patients with a negative 48-hour BH4 loading test in the past with a blood Phe reduction of 20–30% during the 48-hour BH4 loading test loading test or with at least one mutation associated with long-term BH4 responsiveness were eligible for the study. Mutations were selected according to Anjema et al. (2013) including mutations with inconsistent results [7]. Patients had to sign an informed consent.

2.2. BH4 loading test

BH4 was administered orally once daily for 7 days at a dose of 20 mg/kg/day. Patients were instructed to continue their usual diet throughout the test. Blood samples on filter paper were collected at T = −8, 0, 8, 16, 24, 32, 40, 48, 72, 96, 120, 144 and 168 h. With these selection of time points, a repetition of the 48-hour test was combined with an extension to 7 days. Phe was measured prior to the first dose of BH4 at T = 0 as a baseline measurement. All morning samples were taken after an overnight fast.

Preceding the BH4-loading test a 24-hour blood Phe fluctuation was determined by 2 samples (prior to breakfast and 12 h later) on 2 days to help interpret the BH4 loading test results. Also, Phe levels of the previous 6 months and a three-day dietary record were collected to determine the baseline Phe levels and baseline natural protein tolerance.

All blood spots of the BH4 loading test were collected on the same filtration paper (Grade 179 g/m2, Sartorius Stedim DEU) and analysed by LC-MS/MS at the Laboratory of Metabolic Diseases in the UMC Groningen. In order to answer the two secondary questions, the definition of potential BH4 responsiveness was twofold. In order to examine if the duration of the current loading test should be extended to 7 days, potential BH4 responsiveness was defined as ≥30% at ≥1 moment(s) during the entire test. In order to examine if the threshold of 30% should be lowered to 20%, potential BH4 responsiveness was also defined as ≥20% at ≥1 moment(s) within the first 48 h. However, when Phe levels consistently increased above baseline Phe levels (T = 0) after a sufficient reduction within the first 48-hours, patients were considered to be negative. Patients with potential BH4 responsiveness were invited to take part in second phase of the study, the BH4 treatment trial.

2.3. BH4 treatment trial

During the treatment trial, blood samples were collected once to twice weekly after an overnight fast. Both centres used their own filtration paper and samples were analysed at their own laboratories. Natural protein intake, BH4 dosing and AAS were adjusted by the centre's dietician and physician according to usual care. Natural protein was increased with food products by patients' choice. Mean Phe levels during this phase had to stay under the upper target limit (<12 years of age 360 μmoll/L, >12 years of age 600 μmoll/L) according to the Dutch guidelines for PKU of that time that were replaced by the first European PKU guidelines [4,5]. True BH4 responsiveness was defined as ≥30% decrease of Phe levels compared to baseline and/or ≥50% increase in natural protein tolerance according to the Dutch criteria of 2009 [14], as the first European guidelines were not yet published at the start of this study.

2.4. Statistical analysis

Descriptive statistics report the number of potential BH4 responders and true BH4 responders, change in blood Phe levels, natural protein intake and AAS intake. In order to answer the secondary questions, patients were divided according to (1) their inclusion criteria, (2) a Phe reduction between 20 and 30% within the first 48-hours of the test and (3) a Phe reduction ≥30% between day 3 and 7 of the test. IBM SPSS Statistics 23 and Graphpad Prism version 5.03 were used.

3. Results

A total of 24 patients were invited to participate in this study. Twenty-two patients were included in the study and completed the 7-day loading test. Two of these 22 patients were excluded as they did not follow their prescribed diet as usual during the test. The characteristics and results of the remaining 20 patients are shown in Table 1, Figs. 1, 2 and 3a.

3.1. BH4 loading test

In total, twelve patients fulfilled one or both criteria and were considered potential BH4 responders. Consequently, 8 patients were considered to be non-potential BH4 responsive (Table 1). Three of these 8 patients showed ≥20% reduction in blood Phe levels within the first 48 h (21.7, 24.2, and 33.7% reduction). However, as the Phe level increased during the following 5 days above the baseline Phe level still taking BH4, these patients were considered to be non-potential BH4 responders and did not proceed with the treatment trial (Table 1).

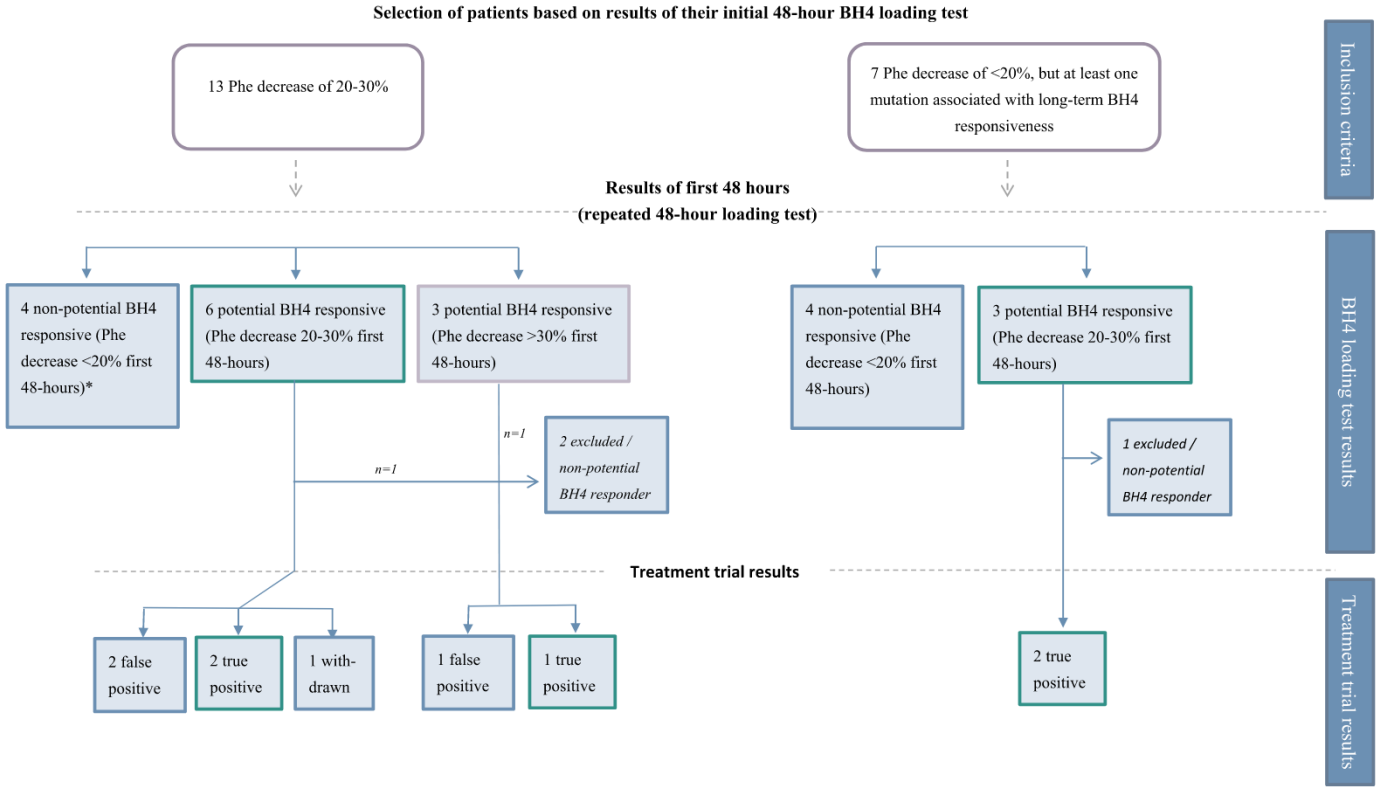
3.2. BH4 treatment trial

Twelve potential BH4 responders underwent a treatment trial. The median duration of the trial was 6 months with a range between 2 and 18 months. Six patients were considered to be true-responders (true positive) according to the study criteria and 5 were considered to be non-true-responders (false positive). Of these 6 true-responders, 5 were able to increase their natural protein tolerance (≥50%) and another one decreased his Phe levels within target range (≥30% compared to baseline) without increasing his natural protein intake (Table 2).

Table 1
Characteristics of negative and false positive results of 7-day BH4 loading test.

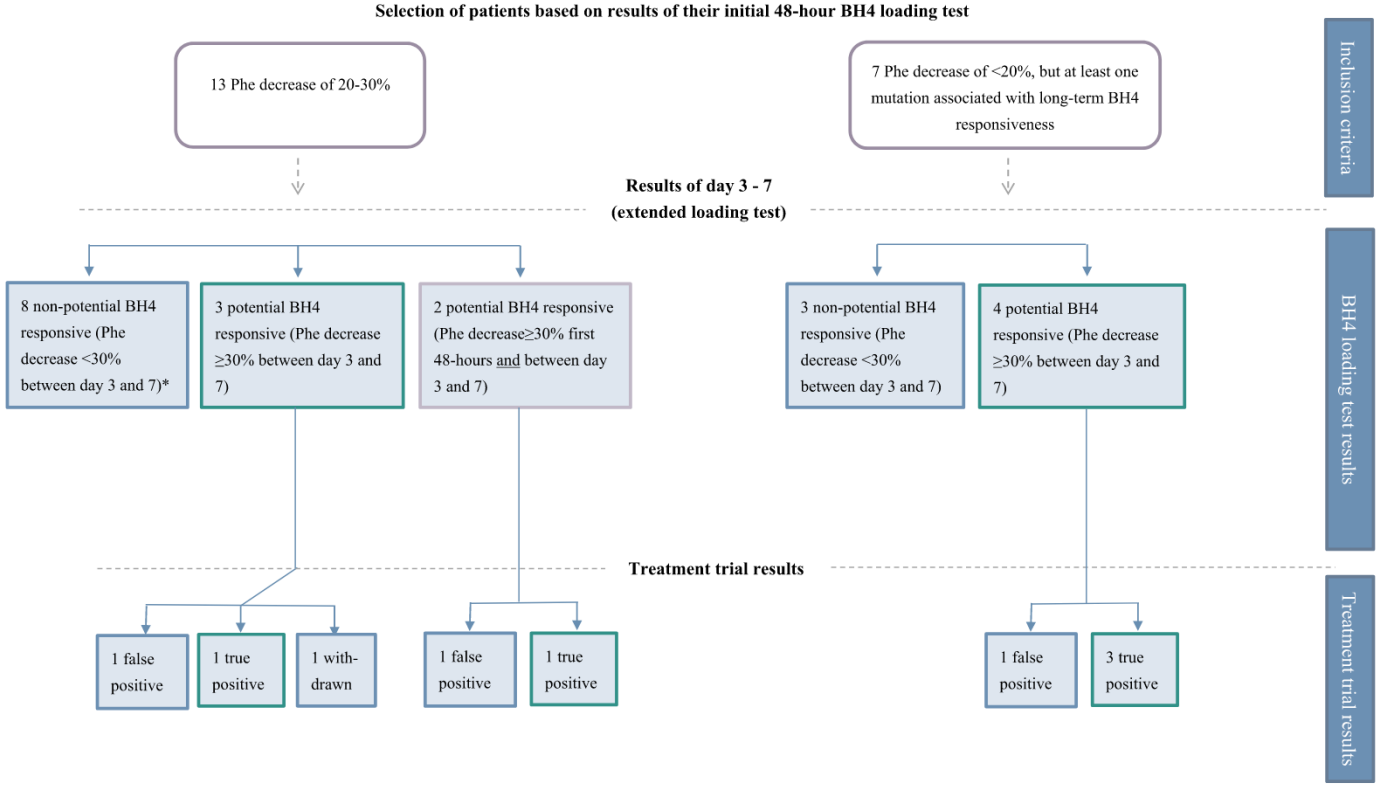
	Patient characteristics				Initial 48-h loading test	7 day BH4 loading test			Genotype		
	Age (years), sex (F/M)	Baseline Phe[a]	Natural protein intake (g)	Protein equivalent AAS (gram)	Max Phe reduction (%)	Max Phe reduction 0-48 h (%)	Max Phe reduction 72-168 h (%)	Highest reduction time	Mutation 1	Mutation 2	BIOPKU database (14-11-2019)
Non-potential BH4 responsive											
1	48 M	604	15,9	69,3	18,1	0,5	0	*T* = 16	p.Y277D[b]	IVS12 + 1(g > a)	0 yes, 4 no, 0 slow
2	21 F	1287	59,6	0	22,1	2,9	0	*T* = 32	p.P281L[b]	IVS7–5(t > c)	0 yes, 1 no, 0 slow
3	29 F	828	6,3	70,3	19,4	10,6	0	*T* = 40	p.P281L[b]	IVS12 + 1(g > a)	0 yes, 13 no, 0 slow
4	20 M	735	40,1	45,0	21,1	15,5	3,2	*T* = 48	p.R243	p.E440	0 yes, 1 no, 0 slow
5	17 F	625	9,6	50,0	27,5	16,3	0	*T* = 24	p.W187	p.R261Q[b]	No records
Non-potential BH4 responsive who initially showed Phe reduction											
6	32 F	429	17,2	60,1	21,8	21,7	0	T = 16	p.P281L[b]	IVS12 + 1(g > a)	0 yes, 13 no, 0 slow
7	19 F	590	12,0	73,5	27,9	33,7	0	T = 16	IVS12 + 1(g > a)	IVS12 + 1(g > a)	0 yes, 13 no, 2 slow
8	28 M	1780	54,2	0	0	24,2	5,1	T = 24	p.R408W[b]	IVS12 + 1(g > a)	0 yes, 40 no, 0 slow
Potential BH4 responder											
9	12 M	260	9,2	42,0	21,7	44,5	34,2	T = 40	p.R261Q[b]	IVS12 + 1(g > a)	0 yes, 24 no, 1 slow
10	23 M	611	11,9	60,0	24,9	11,1	30,5	*T* = 168	IVS2 + 5(g > c)	p.R243	0 yes, 1 no, 0 slow
11	29 M	723	26,6	60,9	24,8	26,4	24,5	T = 32	p.R261Q[b]	p.R261Q[b]	74 yes, 22 no, 4 slow
12	25 M	546	30,4	60,0	6,9	28,7	43,9	*T* = 72	p.R261Q[b]	p.R261Q[b]	74 yes, 22 no, 4 slow
13	15 M	682	19,8	60,0	21,4	20,7	29,7	*T* = 120	IVS2 + 5(g > c)	p.R261Q[b]	5 yes, 3 no, 0 slow
14	30 F	506	9,8	68,0	15,9	28,3	43,2	T = 168	IVS12 + 1(g > a)	p.R261Q[b]	0 yes, 24 no, 1 slow
15	14 M	513	18,5	35,0	0	15,7	38,4	T = 168	p.I65T [c]	p.I65T [c]	11 yes, 2 no, 0 slow
16	20 M	363	13,4	84,0	23,7	23,3	26,9	*T* = 96	L348 V [c]	unknown	/
17	21 F	739	47,7	40,0	14,7	13,5	36,7	T = 168	IVS2 + 5(g > c)	p.R261Q[b]	5 yes, 3 no, 0 slow
18	25 M	545	7,0	90,0	27,9	23,2	32	T = 120	Unknown	Unknown	/
19	14 M	510	26,3	40,0	25,4	26,4	47,8	T = 168	p.R261Q[b]	p.E280K[b]	0 yes, 11 no, 1 slow
20	8 M	468	57,0	18,0	21,5	61,9	49,2	*T* = 8	p.R169G	p.Y414C[c]	p.R169G unknown

AAS: Phe-free L-amino acid supplement.
[a] Mean Phe concentrations during the 6 months before the start of the 7-day BH4 loading test.
[b] Mutation with inconsistent results.
[c] Mutation associated with true-responsiveness.



* 1 of these 4 patients (patient 15) was considered to be a potential responder according to the other definition (≥30% reduction between day 3 and 7) and turned out to be a true-responder.

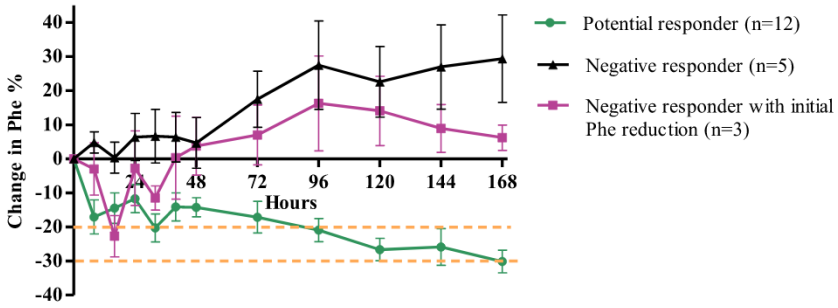
Fig. 1. Results for the first 48-hours of the 7-day BH4 loading test with a lower Phe decrease threshold.



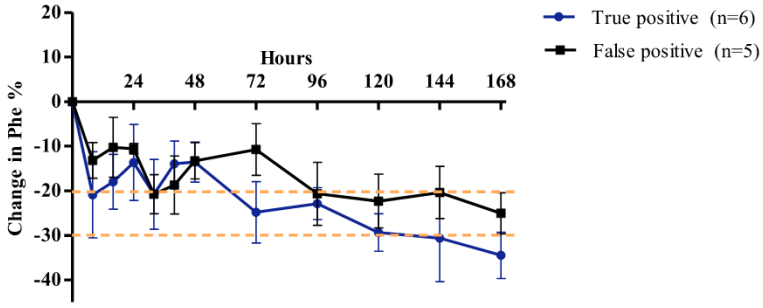
* 1 of these 8 patients (patient 16) was considered to be a potential responder according to the other definition (20-30% reduction in 48 hours) and turned out to be a true-responder.

Fig. 2. Results for the (extension to) 7-day BH4 loading test.

3a) Phe results all patients (mean percentage)



3b) Phe results (mean percentage) of all true-responders (true positives) and non-true-responders (false positives)



3c) Individual loading test results of true-responders (percentage)

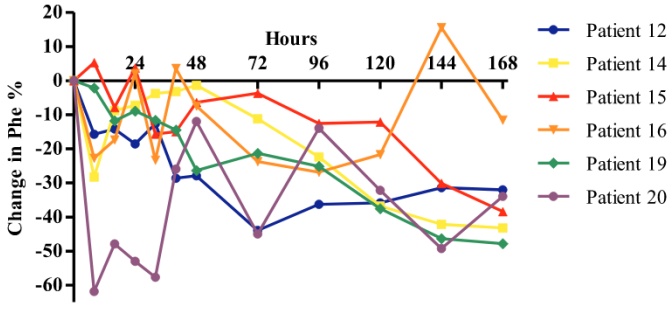


Fig. 3. Results of 7-day BH4 loading test.

Table 2
True-responders treatment trial results.

	Prior to BH4 treatment trial				After BH4 treatment trial				
	Phe levels (μmol/l)[a]	Protein intake (gram)	Protein intake per kg bodyweight	Protein equivalent AAS (gram)	Phe levels (μmol/l)[b]	Protein intake (gram)	Protein intake per kg bodyweight	Protein equivalent AAS (gram)	BH4 dose (mg/kg)
12	546	30,4	0,5	63,3	550 (+1%)	53,2 (+75%)	0,8[d]	33,3(−47%)	20
14	423	8,6	0,1	69	407 (−4%)	22,5(+162%)	0,3	69	20
15	513	18,5	0,4	35	643 (+25%)	33,9 (+83%)	0,7	30 (−14%)	20
16	363	13,4	0,2	84	486 (+34%)	26,6 (+99%)	0,3	70 (−17%)	15
19	510	26,3	0,6	40	No data[c]	39,8–46,3 (+51–76%)	0,8-0,9[d]	40[c]	20[c]
20	474	57,0	1,1[d]	18	331 (−30%)	57,0 (+0%)	1,1[d]	9 (−50%)	20

AAS: Phe-free L-amino acid supplements.
[a] Mean Phe 6 months prior to 7 day loading test.
[b] Mean Phe 3 months after stop treatment trial.
[c] Patient stopped with BH4 treatment as the metabolic team considered the increase of protein as too little benefit.
[d] Intake according to FAO/WHO/UNU safe levels.

One of the 6 true-responders did not proceed with BH4 treatment after the treatment trial as together with the metabolic team it was decided the protein increase was not satisfying. Therefore, the follow-up period was not completed. One patient decided to withdraw during the treatment trial and his test was therefore considered inconclusive.

3.2.1. Treatment trial results for the repeated 48-hour BH4 loading test with a lower threshold

Of the 7 patients who entered the treatment trial because they showed a decrease in Phe between 20 and 30% during the first 48 h, 4 were true-responsive (Fig. 1). One of the in total 6 true-responders was not picked up in the first 48-hours.

3.2.2. Treatment trial results for the 7-day BH4 loading test

Of the 7 patients who entered the treatment trial because they showed a decrease in Phe ≥30% between the extended period day 3 and 7 (but not during the first 48 h), 4 were true-responsive (Fig. 2). Here also, 1 of the 6 true-responders was not picked up.

4. Discussion

The primary aim of this study was to evaluate if the current 48-hour loading test misses BH4-responsive patients, as different loading tests are used around the world to predict true long-term BH4 responsiveness. As BH4-responsive patients are able to liberate their strict dietary treatment or can achieve better metabolic control, these tests need to be sensitive and efficient. In total, we found 6 out of 20 patients to be true-responders, which means the initial 48-hour test was unable to detect some BH4 responders. Secondary, it was examined if 1) the arbitrary threshold of 30% should be lowered to 20%, and 2) if the duration of the test should be increased to 7 days.

To answer the first sub question, we will discuss three results. Firstly, if we look at the first 48-hours of this studies combined BH4 loading test, in total 9 patients showed a reduction of 20–30% Phe in the first 48-hours (Table 1, Fig. 1). Only 4 of these 9 turned out to be true-responders. Secondly, two of these 9 patients did not undergo the treatment trial as their Phe levels increased above baseline Phe level (T0) during the following 5 days of the test. This initial decrease can probably be explained due to within day fluctuations [16], as during the first 48-hours every 8 h a blood sample was retrieved. When we look at the Phe fluctuation data collected before the 7 day loading test, 4 of the 20 patients showed already a difference of >20% (24, 27, 35 and 39%) between the morning and evening sample. So, even without BH4 a decrease of 20% and even 30% is not uncommon. In addition, only 1 of the 3 patients with a Phe reduction of >30% in the first 48-hours of the loading test, turned out to be a true-responder (Fig. 1). Thirdly, on a group level, Fig. 3b shows that a threshold of 20% in 48-hours does not distinguish false positive from true positive responders. Summarizing, a threshold of ≥20% to find potential BH4 responsiveness in a 48-hour BH4 loading test will lead to an increase of false positive responders and not that many true positive responders, and is therefore considered not suitable.

To answer the other question, whether the duration of the 48-hour test should be increased, we only discuss the patients with a Phe reduction of ≥30% between day 3 and 7 (72 and 168 h), but not during the first 2 days (Fig. 2). In total, 7 patients showed a Phe reduction of ≥30% between day 3 and 7. Of these 7 potential responders, 4 turned out to be true-responders, 2 were considered false positive and 1 was withdrawn. Similar to the other definition, 1 of the in total 6 true-responders was missed. In Fig. 3c the particular curve of this patient is shown (patient 16). Perhaps other factors have played a role that consequently influenced the test results negatively.

On a group level an extended test (with a threshold of 30%) seems to be able to distinguish false positives from true-positive responders in most cases (Fig. 3b). Even though the 48-hour BH4 loading test has proven its efficacy, a full week may be necessary to detect all responders if there is a tendency in the 48-hour loading test to be potential responsive. Also, an extended loading test with only measurements each 24 h would probably be less influenced by diurnal fluctuations.

Besides a loading test, BH4 responsiveness can also be predicted with genotype. When comparing our results with the BIOPKU database (www.biopku.org), some important discrepancies were found. In this respect, we should keep in mind however, that BIOPKU database defines BH4 responsiveness based on short loading tests and not on long-term treatment trials.

One patient who was heterozygote for p.R261Q showed no potential responsiveness during the BH4 loading test, although this genotype is strongly associated with BH4 responsiveness. On the opposite, this patient's sibling with the same genotype was found to be BH4 responsive for sure, which requires explanation. Besides this, one patient with a genotype associated with BH4 unresponsiveness (IVS12 + 1(g > a) and p.R261Q) was clearly responsive. These findings confirm that although the genotype can be helpful in predicting BH4 responsiveness, it is not always conclusive [7].

In addition, we found that the patients with mutations P281L, Y277D and R408W, which showed inconsistent results in the cohort of Anjema et al. 2013, were now all considered non-potential responders [7]. The patient with E280K turned out to be a true-responder, however the second mutation was R261Q. Mutation R261Q has been known for inconsistent results [17–19] which was also the case in our study population.

One limitation of this study is that we did not measure quality of life in these patients before and after the BH4 treatment trial. Another important limitation is the definition of true-responsiveness, especially the definition of the amount of natural protein increase which is considered to be enough to continue BH4 treatment. In our study a less strict definition has been used (an increase of natural protein with 50% with blood Phe levels remaining consistently within the target range) than in the recent published European PKU guidelines. The European PKU guidelines define BH4 responsiveness as an increase in natural protein tolerance of ≥100% with blood Phe levels remaining consistently within the target range or as improved metabolic control, defined as >75% of blood Phe levels remaining within target range without any reduction in natural protein intake [4,5].

According to the (protein)definition used in the first European guidelines only 1 patient would have been considered as a true-responder and 1 a borderline true-responder. Two patients (Table 2) would not have been defined as true-responders even though they were able to increase their natural protein intake to WHO safe levels [20]. In practice, there is discussion if the definition of the European guidelines is the most optimal one as patients can really experience their diet in daily life becomes easier with an increase of protein <100%. When we adjust the data of Anjema et al. 2013 to the stricter definition of true-responsiveness, the PPV of the 48-hour loading test drops to 50% instead of 87% in the original publication [21]. To gain more insight in a good definition, long-term follow-up studies comparing outcomes such as quality of life and costs are necessary.

One other important item to address is the financing structure. In the USA and in some centres in Europe the duration of the BH4 loading test is ≥48 h and even up to 28 days. In most of these centres, BH4 is supplied and financed by the manufacturer. In the Netherlands, BH4 is paid by insurance companies as it is regarded as usual care. If this is taken into account, the finding of 1 true-responder and 1 borderline true-responder versus the costs implies that this 7 day BH4 loading test should not replace the 48-hour test for usual care in the Netherlands. Unresponsive patients would receive BH4 unnecessary long. In addition, some potential BH4 responders who turned out to be non-true-responders, experienced the treatment trial as very intensive and disappointing. These treatment trials can be very time-consuming when results are conflicting and inconclusive. Some of them had difficulties to return to their usual restricted diet and control the Phe levels. To shorten and ease the treatment trial, greater steps of protein increase (for example at once +50% or + 100% depending on the goal) could be considered in the form of a supplement of Phe or natural protein [22].

5. Conclusion

Summarizing, although the 48-hour BH4 loading (using a cut-off value of 30% reduction in Phe) does miss some BH4-responsive patients, a cut-off value of 20% should not be used. It is advised a 48-hour BH4 loading test should occur under strict circumstances to limit the impact of diurnal fluctuations. Additionally, an extended BH4 loading test should not replace the current test in the Netherlands but could be offered in cases when there is doubt about the result of a 48-hour BH4 loading test. Cost-effectiveness studies of BH4 treatment in the future would be helpful to define true-responsiveness.

Author contributions

AMJ van Wegberg designed the study, analysed the data, interpreted the results and was the lead writer of the manuscript. RAF Evers interpreted the results and was the second lead writer of the manuscript. FJ van Spronsen designed the study, interpreted the results, co-wrote the manuscript and supervised the project. E van Dam, MC de Vries, MCH Janssen, and MR Heiner-Fokkema co-wrote and approved the manuscript.

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Declaration of Competing Interest

FJ van Spronsen is/was a member of scientific advisory boards for various defects in amino acid metabolism that are supported by BioMarin, SoBi, Arla Food Int, APR, Eurocept, Lucane, Nestle-Codexis Alliance, Orphan Europe, Rivim Medical BV, Origin, Agios, Vivet, and Nutricia International, has received research grants from Nutricia/Danone, SoBi, Alexion, and Merck Serono/Biomarin, Coexis, NPKUA, ESPKU, Dutch PKU research Foundation, Tyrosinemia Foundation and honoraria as a consultant and speaker from Merck Serono, Biomarin, Pluvia Biotech, Nutricia/Danone, and MendeliKABS.

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MC de Vries, MCH Janssen and MR Heiner-Fokkema declare that they have no conflict of interest.

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